ELECTROPHORETIC VARIATION IN NORTHERN DUSKY SALAMANDER *Desmognathus fuscus* POPULATIONS IN EASTERN NORTH AMERICA (Caudata: Plethodontidae)

A Thesis

by

William Minor Hicks

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ABSTRACT

ELECTROPHORETIC VARIATION IN NORTHERN DUSKY SALAMANDER
Desmognathus fuscus
POPULATIONS IN EASTERN NORTH AMERICA
(Caudata: Plethodontidae)

(May 2004)

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An electrophoretic analysis was performed on 336 Northern Dusky salamanders (*Desmognathus fuscus*) from 17 sites in the Eastern US to determine the presence of alternate forms from the piedmont physiographic province. Sites were selected from low (0-239m), medium (240-479m) and, high (>480m) elevations to detect contact zones or range limits if applicable.

Scored gels generated data that helped estimate gene flow between populations. Estimates of heterozygosity among and between populations, probability of Hardy-Weinberg equilibrium, F statistics were all calculated from allele frequencies from each population (county). Dendrograms were also generated to indicate probable relationships between the populations.

Gene flow within populations was mostly as predicted with populations breeding in Hardy-Weinberg equilibrium. F_{ST} and F_{IT} values indicate a reduction in heterozygosity

among populations and within the whole population (0.17 to 1.0 and 0.20 to 0.76, respectively) Dendrograms indicated five distinct groups with genetic distances from D = 0.13 to D = 0.68 indicating several species may be present. Three undescribed species of *Desmognathus* were in the North Carolina piedmont.

ACKNOWLEDGEMENTS

The term "thanks" is entirely insufficient to express my gratitude for the help I received from Wayne Van Devender. With patience, guidance, knowledge and of course, endless pots of coffee, he has helped me refine my scientific skills. For this I am truly grateful.

I thank the following for their help in the field collections for this study: Andy Baldwin, Brian Ball, Leigh Anne Harris, Lillian Hicks, Carl Ray Morgan, Steve Kahn, Kevin Lapp, John Lester, John MacGregor, Mike Seils, Wayne Van Devender and the Spring 1996 Mammology class. Everyone mentioned added much to the enjoyment of the field work.

I am indebted to Kim Hicks, Paul Johns, Peg Mulvey, and Steve Tilley for their help with gel recipes, gel scoring and assistance with the lab work. Their skill and guidance in the SREL "Electro lab" greatly aided this study. I could not have completed this project without the support of Carl and Sandy Morgan, my father Bill Hicks, and my committee, all of whom showed great patience and support throughout this research.

This research was supported by grants from the Cratis D. Williams Graduate School, the Graduate Student Association Senate, and the North Carolina Herpetological Society. Finally, I thank my family for their continued support, especially my wife Kim and my son Eli, who were a great source of motivation.

DEDICATION

"He who breaks a thing to find out what it is has left the path of wisdom."

---- J.R.R. Tolkien

Alas, so many broken salamanders! May this work one day assist them. This thesis is dedicated to my wife, Kim whose love, support and technical assistance made this study possible.

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Introduction

Salamanders are model organisms for systematists and evolutionary biologists because of abundance, ease of collection, and variability across their ranges (Petranka, 1998). Several species having large ranges have been shown to have particularly interesting systematic histories over the last few decades. Each species is now recognized as being composed of several smaller units, which are genetically isolated from each other. The former *Plethodon glutinosus* (Slimy Salamander) is now recognized as a species complex including at least 16 described species (Highton, 1989; Highton et al., 1989; Highton and Peabody, 2000). The differences in these groups are revealed by electrophoresis, microcompliment fixation, and even morphology. Populations once known as *Plethodon jordani* (Appalachian Woodland Salamander) are now a complex of seven species which are nested within the *Plethodon glutinosus* complex (Highton and Peabody, 2000). Slender salamanders in the Genus *Batrachoseps* are placed in 15 species instead of three (Wake and Jockush, 2000). In fact, these species form six monophyletic groups. The Two-Lined Salamander (*Eurycea bislineata*) is now described as three separate species (Jacobs, 1987). In the Appalachian Mountains the *Desmognathus ochrophaeus* complex consists of four species (Tilley and Mahoney, 1996). More species are described in these groups every year

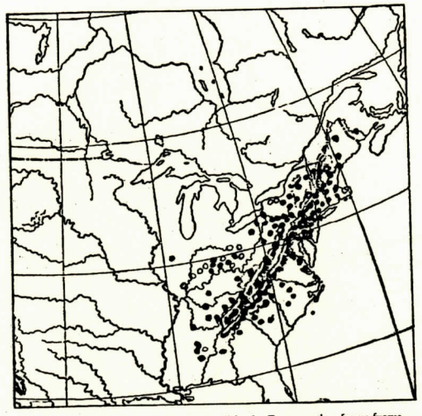
The Northern Dusky Salamander, *Desmognathus fuscus* (*sensu lato*), has a geographic range extending from New Brunswick, Canada to southern Louisiana and Alabama. The western edge of the range is east of the Mississippi River except for areas in western

Louisiana, Arkansas and Kentucky. Northern Dusky Salamanders do not occupy the coastal plain physiographic province from southern Virginia to western Florida, an area occupied by the closely related *Desmognathus auriculatus* (Southern Dusky Salamander) (Conant and Collins, 1998; Petranka, 1998). *D. fuscus* is found from high elevations of the Blue Ridge Mountains to low coastal areas (Tilley, 1981; Conant and Collins, 1998) in at least nine physiographic provinces and over 40 river drainages (Griffin, *fide* Wright and Frey, 1965). For all these reasons, *Desmognathus fuscus* is probably composed of several additional species.

The history of *Desmognathus fuscus* seems to include several waves of description, synonymy, and subdivision. The early days of *Desmognathus* classification were chaotic because scientists lacked the extensive collections available today (Grobman 1950). It is not even clear who described *D. fuscus* or when. Green (1818, *fide* Dunn 1926) may have described *Desmognathus fuscus* as both *Salamandra nigra* and *Salamandra fusca*; but he failed to designate holotypes or type localities for either. As a result, authorities dispute what name to use today (Dunn, 1926; Bishop, 1947; Petranka, 1998; and Frost, 2002). Rafinesque (1830, *fide* Dunn 1926) added to the problem by describing *Triturus fuscus* for the same real species. There are no extant holotypes associated with this description either. By the end of the 1800's at least seven additional names were used for *D. fuscus* (Frost, 2002).

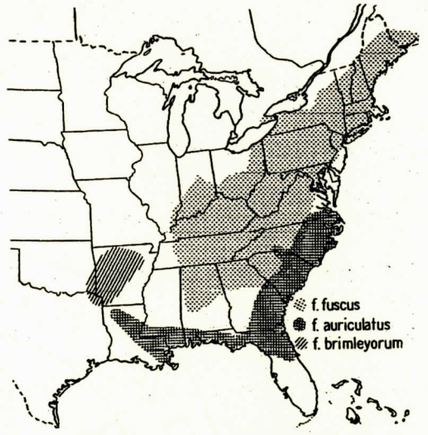
The early nomenclatural history of *Desmognathus* seemed turbulent; however, by 1926, our modern concepts of the Family Plethodontidae and Genus *Desmognathus* had been roughly settled by Dunn's The Salamanders of the Family Plethodontidae (1926). He published the first range map for *Desmognathus fuscus* (Figure 1, panel A). Bishop's Handbook of Salamanders (1947) refined the map and added ranges for two subspecies

Figure 1. Maps showing historical distribution of *Desmognathus fuscus*.



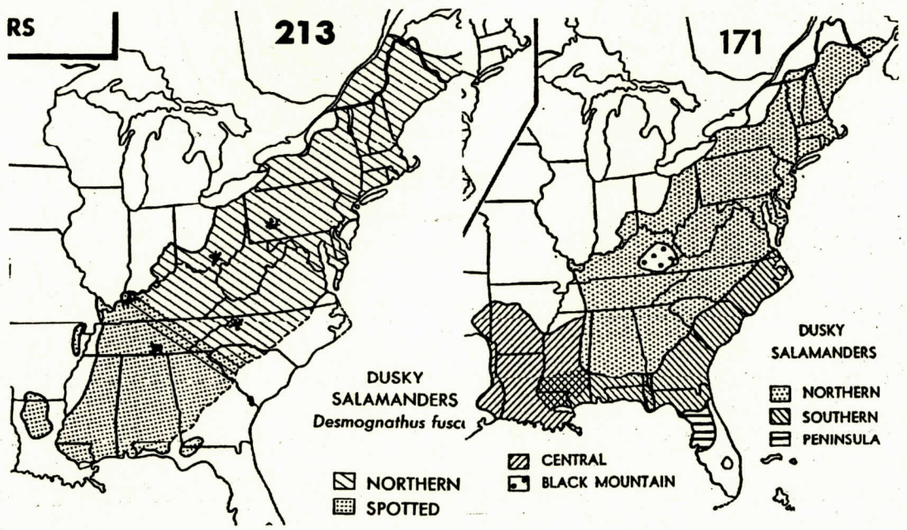
4. Eastern North America, showing localities for Desmognathus fuscus fuscus.

A. Distribution according to Dunn (1926).



MAP 23.—Distribution of the subspecies of *Desmognathus fuscus*.

B. Distribution according to Bishop (1947).



C. Distribution according to Conant (1959).

D. Distribution according to Conant and Collins (1989).

(*D. f. auriculatus* and *D. f. brimleyorum*) (Figure 1, panel B). An additional subspecies, the Spotted Dusky Salamander (*Desmognathus fuscus conanti*), was described (Rossman, 1959) for a southern form of *Desmognathus fuscus* (Figure 1, panel C). Distributions of *Desmognathus fuscus* were partitioned to include groups in Kentucky (*D. welteri*). (Barbour, 1950), Western North Carolina, (*D. santeetlah*) (Tilley, 1981) and the Florida Panhandle (*D. apalachicolae*) (Means and Karlin, 1989) (Figure 1, panel D).

Additional species of *Desmognathus fuscus* will be recognized in other areas within its range. Systematic work has left the piedmont of North Carolina mostly untouched but there seems to be an alternate form from this region (Van Devender, pers. comm.; Braswell, pers. comm.). Based on both morphology and habitat differences, *Desmognathus fuscus* appears to be more than one form.

This study will attempt to determine if alternate forms of *Desmognathus fuscus* exist in the southeastern United States and if any of these correspond to distributions according to Petranka (1998). Collections of representative populations from high and low elevations will be made and analyzed electrophoretically. The populations will be characterized genetically. Salamanders will be grouped according to allele frequencies. Relationships among groups will be determined with UPGMA dendrograms, and tentative distributions of each group will be mapped. For each group a biogeographic hypothesis will be presented to indicate possible causes for each population's distribution.

Materials and Methods

Collection sites were selected to form transects (Figure 2, Table 1, Appendix 1). The first transect extended from Pulaski County, KY in the northwest to Johnston County, NC in the southeast. The second transect extended from Rockingham County NC southwest to Tuscaloosa County AL. Specific sites were located where seeps or small streams crossed public roads. Data recorded for each site included: state, county, elevation, drainage system, latitude, longitude, habitat description, and number of salamanders collected. Salamanders were collected between October 1996 and February 1998 and at each site, samples of 25 salamanders (Highton, 1977, 1989; Tilley, 1977) were collected by hand. If necessary, sites were visited several times to complete the sample, and several samples were never completed. A series (n = 2) of *Desmognathus fuscus* was also obtained from Massachusetts through Dr. Steve Tilley of Smith college. A sample of *Desmognathus orestes* was collected on the Appalachian State University campus for use as an outgroup in the genetic analyses.

Salamanders were kept on ice in freezer boxes or plastic bags during transport to the laboratory at ASU, where they were maintained at near 10° Celsius (C) until processed. Salamanders were sacrificed by submersion in 2% phenoxyethanol for at least 20 minutes. Liver, heart, and muscle tissue were removed from each individual, placed in a 1.5 ml snap cap container with several drops of deionized water, and stored at -60° (C) until used. Salamanders less than 25 mm in total length were too small for tissue removal and were used in their entirety as one tissue sample. Voucher specimens were preserved

Figure 2. Map of collection sites. Numbered sites indicate populations analyzed electrophoretically, and red circles indicate complete collections without electrophoresis. See Table 1 and Appendix 1 for specific data for each site. Population 10 is the outgroup, Desmognathus orestes, and occupies the same position as population 2.

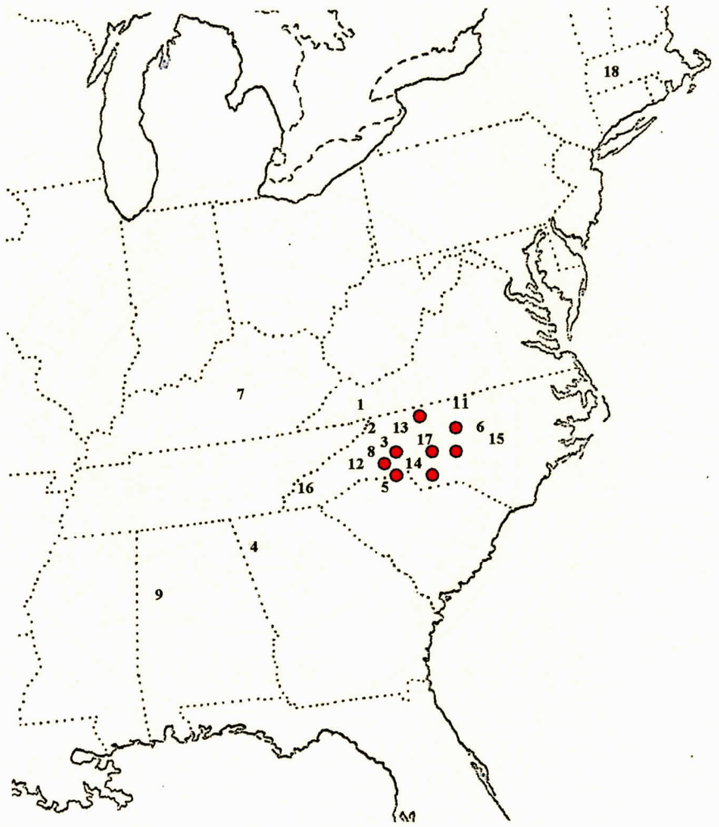


Table 1. Locality data for *Desmognathus fuscus* collections used in electrophoretic analyses. Voucher specimens for each series were deposited in the Appalachian State University collection.

Population Number	n	State	County	Elevation (km)
1	25	NC	Washington	488
2	25	NC	Watauga	1016
3	25	NC	Caldwell	519
4	25	GA	Walker	168
5	25	SC	Cherokee	82
6	25	NC	Wake	111
7	25	KY	Pulaski	366
8	25	NC	Burke	572
9	24	AL	Tuscaloosa	76
10	25	NC	Watauga	1035
11	26	NC	Rockingham	277
12	25	NC	McDowell	457
13	26	NC	Wilkes	398
14	24	NC	Gaston	251
15	19	NC	Johnston	47
16	20	NC	Graham	977
17	20	NC	Iredell	274
18	2	MA	Hampton	650

in 10% formaldehyde, catalogued into the Appalachian State University (APPSU) collection of amphibians, reptiles and mammals, and stored in 70% ethanol. Frozen tissues were placed in liquid nitrogen and transported to Savannah River Ecology Laboratory near Aiken, South Carolina for electrophoresis. After tissues were thawed, one drop of grinding solution was added and tissues were macerated manually for 30 seconds with a sharpened glass rod then centrifuged at 2500 rpm for two minutes Potato starch gels were prepared according to standard procedures (Richardson et al., 1986). Protein extracts were absorbed into filter paper wicks (2x10 mm) by saturating them with supernatant after tissue homogenization. Tissue homogenates were returned to the -60° C freezer immediately.

Wicks were placed in a transverse slit cut 30 mm from the negative (cathode) end of the gel. To resolve questions about whether bands on different gels were identical and to minimize the need for later side-by-side comparisons, each gel contained extracts from three populations (n= 10, 5, and10); and each population was represented on three gels. When sufficient individuals were available, ten samples from one site were loaded next to five from another site; and these were loaded next to ten more from a third site. Standards from population 17 were added between each locality. Tissues and homogenates were transported to ASU in liquid nitrogen and stored at -62° C.

Several combinations of buffers (gel, anode, and cathode) were used in an effort to resolve twenty two presumptive loci (Tilley, 1996) (Table 2). Tris-citrate (pH = 8.0) buffer was used to resolve aconitate hydrotase (ACON), isocitrate dehydrogenase (ICD), malic enzyme (ME), malate dehydrogenase (MDH), and glutamate dehydrogenase (GDH). Poulik buffer was used to resolve lactate dehydrogenase-2 (LDH2), leucine-glycine-glycine peptidase (LGG-PEP), and fumaric acid (FUM). Tris-EDTA-borate (pH

Table 2. Buffer systems used to resolve presumptive loci of enzymes.

Buffer System	Locus
Tris-Citrate , pH = 8.0	Isocitrate dehydrogenase 2 (ICD2)
	Malate dehydrogenase 2 (MDH2)
Poulik	Leucine-glycine-glycine peptidase (LGG-PEP)
Lithium Hydroxide (LIOH)	Mannose-6-phosphate (MPI)

= 9.1) was used to resolve glutamate dehydrogenase (GLUD), glycerol-3- phosphate dehydrogenase (AGPDH), glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 6 phosphogluconic dehydrogenase (6PGDH) and aspartate amino transferase-1 (AAT-1). Tris-citrate (pH = 6.7) was used to resolve adenylate kinase (AK), creatine kinase (CK), and aspartate transferase-2 (AAT-2). Tris-versine-borate buffer (pH = 8.0) was used to resolve hydroxybuterate dehydrogenase (HBDH), and superoxide dismutase (SOD). Lithium hydroxide buffer was used to resolve glucose-6-phosphate isomerase (GPI), lactate dehydrogenase-1 (LDH-1), and mannose-6-phosphate (MPI).

Each gel was subjected to a constant voltage for a period of time appropriate to the buffer system used (Appendix 2). Gels were sliced horizontally with a thin wire to an approximate thickness of 1mm produce up to four separate gels for staining. Each slice

was placed in a tray where a specific stain was added. In each case, the relevant enzyme catalyzed a reaction that led to the deposition of a colored substance in the gel. The colored substance was usually seen as a distinct band revealing the position of the protein. The gel was photographed and scored immediately. Each individual was evaluated based on the bands present in its lane and assigned to a tentative genotype. All bands were scored based on relative distance from the origin, giving scores such as "slow", "medium" or "fast". An individual with only one band was scored as homozygous for a particular allele based on its relative mobility. Similarly, individuals were scored as heterozygous for two alleles if they exhibited two or more bands for a particular locus. Each gel was scored by two or three additional observers. Differences in scorings amongst observers were rare and were resolved to produce general consensus. Gel photographs were scored again at ASU. Only gels that were clearly scorable from photographs were used in the analyses. This process reduced the number of analysis to four loci, ICD II, MDH II, IGG, and MPI.

Genotypic data were entered into Microsoft Word and transferred to the programs GENE (May et al., 1981) and PopGene (Yeh et al., 1986). GENE and PopGene provide measures of genetic variation within each population, compared these measures to those expected under Hardy-Weinberg equilibrium, and produced UPGMA dendrograms showing relationships among the populations based on Nei's (1972) measure of genetic distance. Statistics calculated for each population included heterozygosity measures and values of Fis, (a measure of loss of heterozygosity within a population), Fit, (a measure of loss of heterozygosity across populations) and Fst, (a measure of loss of heterozygosity due to allele frequency differences among all populations). Each population was tested for Hardy-Weinberg equilibrium using χ^2 and G-statistics. Significant levels were $P<$

0.05 for significant (*) and $P< 0.01$ for highly significant (**). PopGene program (Yeh et al., 1999) was used to validate the older GENE program, calculate statistics for allele frequencies and genetic distances and to produce clearer trees.

Windows Excel was used to convert allele frequencies into pie charts for each locus and population. Pie charts were aligned according to calculated groups based on Nei's distances to display graphically overall variability. Since few populations had complete data for the MPI locus, it was removed from the data set and all calculations were repeated in GENE and PopGene.

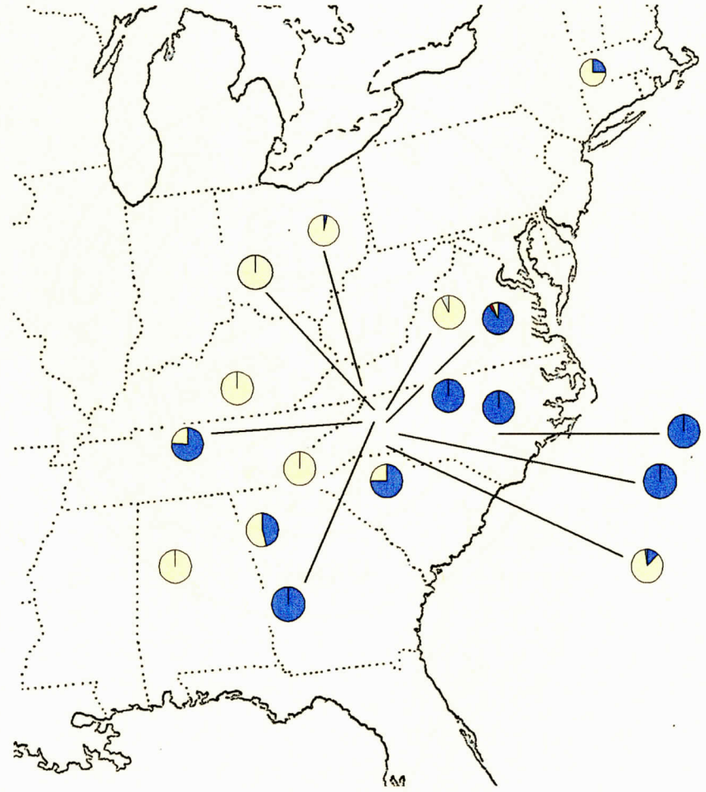
In order to interpret genetic data, geographical data such as elevation, river drainages, physiographic provinces, linear distance, and distribution maps of other salamander species were examined for correspondence with major branches of the UPGMA dendrograms.

Results

A total of 756 salamanders were collected at 36 sites in eight states (Appendix 1). Several criteria were used for the inclusion of populations in subsequent analyses. Several populations, including all collections of *Desmognathus auriculatus* and *D. santeetlah,* included too few individuals for analysis for electrophoresis. As funding was insufficient to analyze all populations, only those representing the overall boundaries of the distribution and major eco-geographic regions were included. Duplicate populations in a geographic province were sometimes excluded. For example, Guilford County NC was excluded because of its proximity to the Rockingham County sample. However, Wake and Johnston counties in NC were on the eastern edge of the range for *D. fuscus*, so both were included. Eighteen populations, including standards from Massachusetts and the outgroup, were used in final analyses. Three buffer systems (TEB 9.1, TC 6.7 and TVB 8.0) produced no usable results and were removed from analyses. Photographic scoring further reduced the number of usable loci to four (Appendix 3).

Four alleles were found for locus ICD 2. The fastest allele, represented in blue in Figure 3, was most common in most southeastern populations but occurred in Massachusetts as well. The slowest form, (Figure 3) was most common in the western half of the study area and in Massachusetts. Two populations (Wilkes and Gaston counties) between the eastern and western groups had a rare allele with intermediate mobility, represented in light blue (Figure 3). The Caldwell County population had a different intermediate rare allele, represented in red (Figure 3).

Figure 3. Distribution of four ICD2 alleles among populations of *Desmognathus fuscus* in eastern US. The slower form (ICD2-1) is represented with blue. The two intermediate forms (ICD2-2 and ICD2-3) are represented with red and light blue respectively. The faster allele (ICD2-4) is represented in yellow. Base map is a modification from Hubricht (1985).



Three alleles were detected for the MDH 2 locus. The fastest allele, represented in blue (Figure 4), was fixed in all but three populations. The slowest allele, represented in yellow (Figure 4), occurred in low frequency in Wilkes and Iredell counties. Caldwell County had a relatively common unique allele.

Two alleles were detected for the MPI locus. The faster allele, represented in red (Figure 5), was fixed in three populations. The slower allele, represented in blue (Figure 5), was fixed in Caldwell County. This locus was scored for only four populations.

Four alleles were detected for the LGG-PEP locus. The slowest allele, represented in (Figure 6), was most common in northern and western populations and in Wake and Johnston counties. The slowest allele, represented in light blue (Figure 6), occurred only in McDowell County. An intermediate form, represented in red (Figure 6), was most common in four southeastern populations. An additional intermediate form, represented by dark blue in Figure 6, was found in populations from Tuscaloosa Co., AL to Wake County, NC.

Some populations were not in Hardy-Weinberg equilibrium for some loci. MDH 2 and MPI loci were always in Hardy-Weinberg equilibrium, but other loci sometimes differed from Hardy-Weinberg equilibrium. In 56 χ^2 and G-tests (PopGene) for Hardy-Weinberg equilibrium for each combination of locus and population, only eight loci deviated significantly from expected values. Gaston County, NC and Cherokee County, SC differed significantly (*) from Hardy-Weinberg equilibrium for the ICD 2 locus. Deviation from Hardy-Weinberg equilibrium was highly significant (**) in Wilkes County, NC for this locus. For the LGG-PEP locus, five populations were statistically

Figure 4. Distribution of three MDH2 alleles among populations of *Desmognathus fuscus* in eastern US. The slower form (MDH2-1) is represented with blue. An intermediate form (MDH2-2) is represented with red and the fastest allele (MDH2-3) is represented with yellow. Base map is a modification from Hubricht (1985).

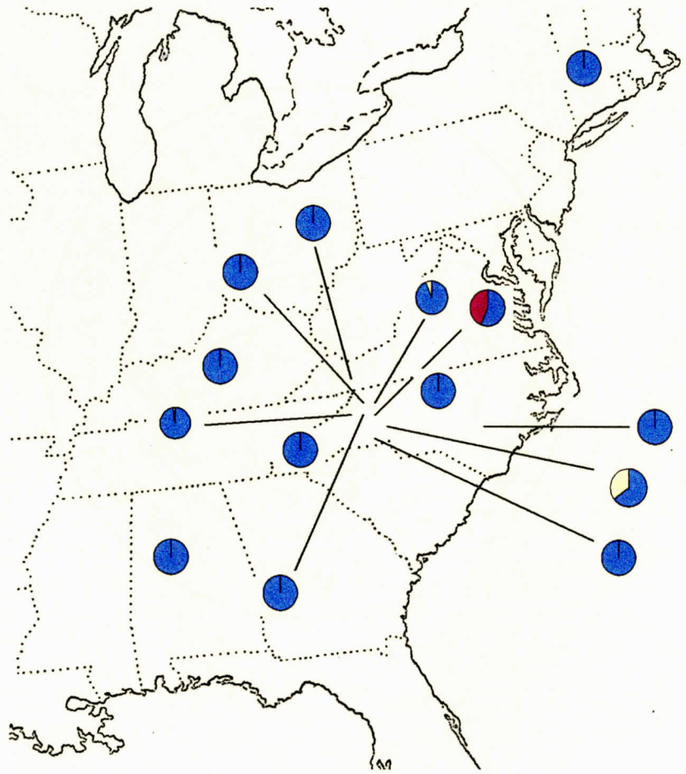


Figure 5. Distribution of two MPI alleles among populations of *Desmognathus fuscus* in eastern US. The fastest form (MPI-1) is represented with blue and the slowest (MPI-2) is represented with red. Base map is a modification from Hubricht (1985)

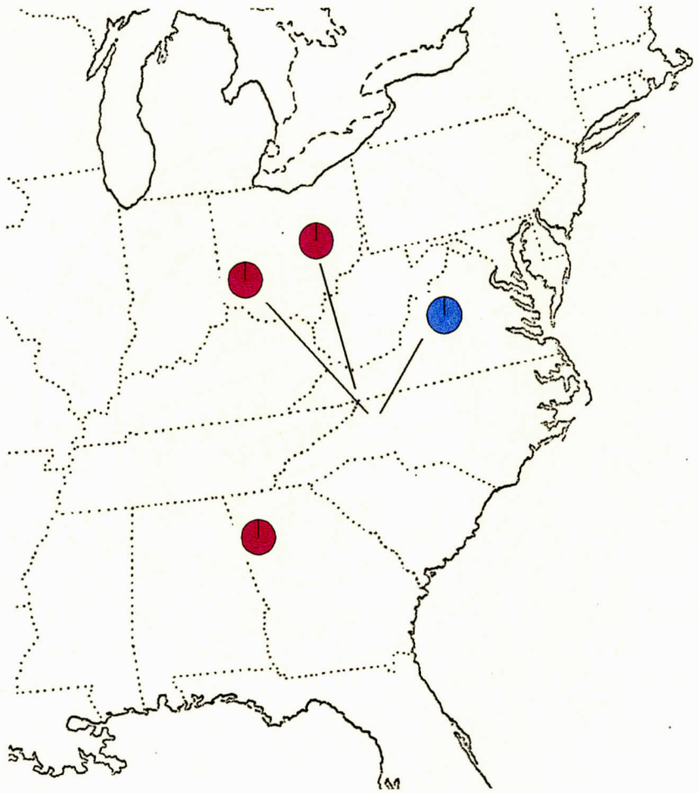
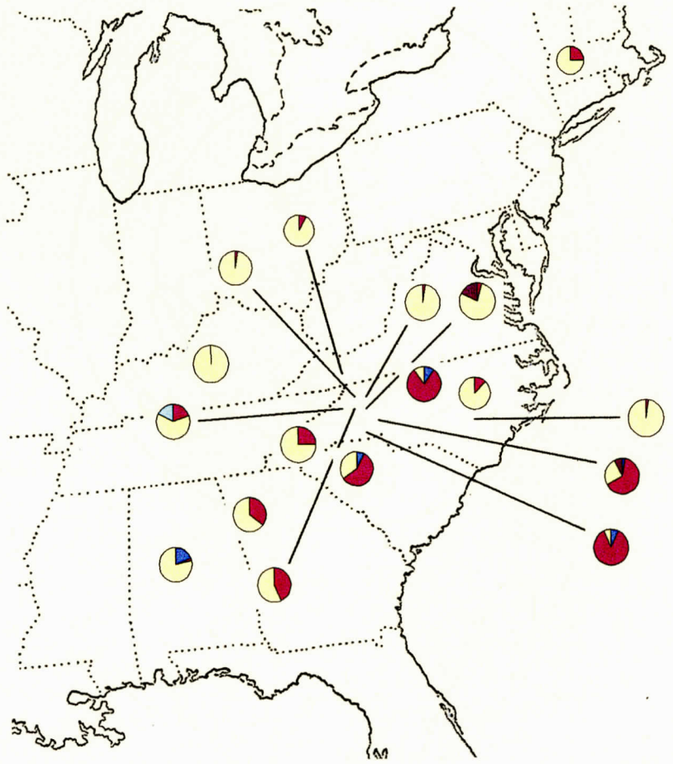


Figure 6. Distribution of four LGG-PEP alleles among populations of *Desmognathus fuscus* in eastern US. The slowest form (LGG-PEP-1) is represented with blue. Four intermediate forms (LGG-PEP-2, 3 and 4) are represented with red, yellow and light blue respectively. The fastest form(LGG-PEP) is represented with dark red. Base map is a modification from Hubricht (1985).



different from Hardy-Weinberg equilibrium: Walker County, GA (**), and Wake (**), Rockingham (**), McDowell (**), and Iredell (*) counties, NC. In each case of deviation from Hardy-Weinberg equilibrium, populations had too few heterozygotes and too many homozygotes for rare alleles.

Analysis of frequency of heterozygotes in each population extended the χ^2 results to stratified groups of populations. Heterozygosity values (H_I, Table 3) averaged 10.7 percent and varied between 0.00 and 0.2312. Comparisons of observed frequencies of heterozygotes with those predicted for both structured and unstructured populations (H_S and H_T, respectively, in Table 3) provided F statistics for detecting reduced heterozygosity associated with each structured level of the whole population. Loss of heterozygosity associated with inbreeding within the total population was detected when F_{IT} (= $(H_T- H_I)/H_T$) was higher than 0.05. Heterozygotes were rarer than expected for all loci in an unstructured population (F_{IT} from 0.205 to 0.761, Table 4). Loss of heterozygosity associated with inbreeding within local populations was detected when F_{IS} (= $(Hs- H_I)/Hs$) was higher than 0.05 (Wright, 1978 *fide* Hartl, 1988), which only occurred for the LGG-PEP locus (F_{IS} = 0.217, Table 4). For the LGG-PEP locus, heterozygotes were rarer than expected within populations. Loss of heterozygotes associated with recognition of subpopulations was detectable when F_{ST} (= $(H_T-H_S)/H_T$) exceeded 0.05 and was considered very important when F_{ST} exceeded 0.25. F_{ST} values ranged from 0.17 to 1.00 (Table 4); so all loci had fewer heterozygotes than expected for a single panmictic population, and separation of population was supported.

Table 3. Heterozygosity in *Desmognathus* populations as calculated by GENE.

Locus	Sample Size	Obs. Het. (H_I)	Exp. Het (H_T)	Ave. Het (H_S)
ICDII	752	0.0904	0.5064	0.1268
MDHII	580	0.1069	0.1525	0.0838
MPI	150	0.0000	0.4474	0.0000
LGG	666	0.2312	0.5040	0.3171
Mean	537	0.1071	0.4026	0.1319

Table 4. F-statistics for *Desmognathus* populations as calculated by GENE.

Locus	Sample Size	F_{IS}	F_{IT}	F_{ST}
ICD2	752	0.0488	0.7613	0.7490
MDH2	580	0.0421	0.2050	0.1710
MPI	150	0.0000	1.0000	1.000
LGG	666	0.2170	0.5023	0.3644
Mean	537	0.0770	0.7501	0.3210

Genetic similarity among populations was depicted in UPGMA dendrograms based on Nei's (1972) genetic distances (Figures 7 and 8). The dendrogram in Figure 7 was calculated using all four loci; while the one in Figure 8 did not include the MPI locus, which was removed due to missing data in 14 of 18 populations. Nei's genetic distance and identity values were calculated for four loci data set (Table 5) and for the data for three loci (Table 6). Five distinct groups were evident in each dendrogram.

When genetic distances were calculated using all four loci, values ranged from D = 0.0010 between Watauga and Washington counties to D = 1.98 between Wake and Gaston counties (Table 3). Similarity values ranged from I = 0.1308 for *Desmognathus*

Table6. Nei's genetic distances and similarities without MPI for *Desmognathus* populations. Identrity measures are above and to the right of the diagonal; genetic distances are below and to the left.

pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	***	0.9988	0.6190	0.6806	0.3666	0.3855	0.9980	0.6062	0.9890	0.8132	0.4410	0.7684	0.9888	0.7415	0.6783	0.9890	0.4098	0.9765
2	0.0012	****	0.6071	0.6705	0.3361	0.3739	0.9999	0.5747	0.9884	0.7980	0.3980	0.7456	0.9880	0.7125	0.6613	0.9832	0.3706	0.9650
3	0.4797	0.4991	****	0.7012	0.6633	0.8727	0.6109	0.8473	0.5646	0.317	0.6408	0.8620	0.5761	0.3390	0.9307	0.5556	0.7077	0.6930
4	0.3848	0.3997	0.3549	****	0.8533	0.7812	0.6692	0.6040	0.6339	0.5962	0.4850	0.6498	0.6416	0.5393	0.6232	0.6723	0.5837	0.7126
5	1.0035	1.0905	0.4105	0.1586	****	0.8098	0.3306	0.7252	0.3245	0.4338	0.7385	0.6430	0.3262	0.5071	0.6166	0.3877	0.8169	0.4988
6	0.9533	0.9837	0.1362	0.2469	0.2110	****	0.3785	0.7337	0.3176	0.1308	0.5455	0.6972	0.3318	0.1381	0.8095	0.3179	0.6826	0.4683
7	0.0020	0.0001	0.4929	0.4016	1.1067	0.9715	****	0.5716	0.9872	0.7894	0.3894	0.7440	0.9868	0.7006	0.6636	0.9800	0.3641	0.9623
8	0.5006	0.5539	0.1657	0.5041	0.3213	0.3097	0.5594	****	0.5645	0.4695	0.9298	0.9596	0.5744	0.6130	0.9428	0.5977	0.9170	0.7508
9	0.0111	0.0116	0.5717	0.4559	1.1254	1.1471	0.0129	0.5719	****	0.8492	0.4203	0.7352	0.9836	0.7522	0.6311	0.9832	0.3728	0.9597
10	0.2068	0.2256	1.1466	0.5171	0.8352	2.0338	0.2365	0.7561	0.1635	****	0.5066	0.5808	0.8268	0.9144	0.3840	0.8760	0.4733	0.8273
11	0.8187	0.9213	0.4450	0.7236	0.3031	0.6060	0.9432	0.0727	0.8669	0.6800	****	0.8367	0.4205	0.7095	0.7564	0.4825	0.9442	0.6234
12	0.2635	0.2936	0.1485	0.4311	0.4416	0.3607	0.2957	0.0413	0.3076	0.5434	0.1783	****	0.7421	0.6562	0.9567	0.7486	0.8242	0.8690
13	0.0112	0.0121	0.5515	0.4438	1.1201	1.1032	0.0133	0.5544	0.0166	0.1902	0.8663	0.2983	****	0.7459	0.6414	0.9821	0.3955	0.9615
14	0.2991	0.3389	1.0818	0.6175	0.6791	1.9801	0.3558	0.4894	0.2847	0.0895	0.3432	0.4213	0.2932	****	0.4461	0.8270	0.6006	0.8164
15	0.3882	0.4136	0.0718	0.4728	0.4835	0.2113	0.4101	0.0589	0.4602	0.9572	0.2792	0.0443	0.4441	0.8071	****	0.6243	0.7797	0.7714
16	0.0110	0.0169	0.5876	0.3971	0.9476	1.1459	0.0202	0.5146	0.0169	0.1324	0.7288	0.2895	0.0181	0.1900	0.4712	****	0.4325	0.9773
17	0.8922	0.9927	0.3457	0.5383	0.2022	0.3818	1.0102	0.0867	0.9867	0.7480	0.0574	0.1934	0.9275	0.5098	0.2489	0.8381	****	0.5840
18	0.0238	0.0356	0.3668	0.3389	0.6955	0.7587	0.0385	0.2866	0.0411	0.1896	0.4726	0.1404	0.0392	0.2028	0.2596	0.0230	0.5379	****

Table 5. Nei's genetic distances and similarities for *Desmognathus* populations using four loci. Identity measures are above and to the right of the diagonal; genetic distances are below and to the left.

pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	****	0.9990	0.4423	0.5835	0.3143	0.3305	0.8555	0.5197	0.8479	0.6971	0.3781	0.6587	0.8477	0.6357	0.5815	0.8479	0.3513	0.8372
2	0.0010	****	0.4374	0.5796	0.2905	0.3232	0.8643	0.4968	0.8544	0.6898	0.3440	0.6445	0.8540	0.6159	0.5716	0.8499	0.3203	0.8342
3	0.8158	0.8269	****	0.5845	0.5529	0.7274	0.5092	0.7063	0.4706	0.2648	0.5341	0.7185	0.4802	0.2825	0.7758	0.4631	0.5899	0.5776
4	0.5387	0.5454	0.5370	****	0.8533	0.7812	0.6692	0.6040	0.6339	0.5962	0.4850	0.6498	0.6416	0.5393	0.6232	0.6723	0.5837	0.7126
5	1.1575	1.2362	0.5926	0.1586	****	0.8098	0.3306	0.7252	0.3245	0.4338	0.7385	0.6430	0.3262	0.5071	0.6166	0.3877	0.8169	0.4988
6	1.1073	1.1294	0.3183	0.2469	0.2110	****	0.3785	0.7337	0.3176	0.1308	0.5455	0.6972	0.3318	0.1381	0.8095	0.3179	0.6826	0.4683
7	0.1560	0.1459	0.6750	0.4016	1.1067	0.9715	****	0.5716	0.9872	0.7894	0.3894	0.7440	0.9868	0.7006	0.6636	0.9800	0.3641	0.9623
8	0.6546	0.6996	0.3478	0.5041	0.3213	0.3097	0.5594	****	0.5645	0.4695	0.9298	0.9596	0.5744	0.6130	0.9428	0.5977	0.9170	0.7508
9	0.1650	0.1574	0.7538	0.4559	1.1254	1.1471	0.0129	0.5719	****	0.8492	0.4203	0.7352	0.9836	0.7522	0.6311	0.9832	0.3728	0.9597
10	0.3608	0.3713	1.3287	0.5171	0.8352	2.0338	0.2365	0.7561	0.1635	****	0.5066	0.5808	0.8268	0.9144	0.3840	0.8760	0.4733	0.8273
11	0.9727	1.0671	0.6271	0.7236	0.3031	0.6060	0.9432	0.0727	0.8669	0.6800	****	0.8367	0.4205	0.7095	0.7564	0.4825	0.9442	0.6234
12	0.4175	0.4393	0.3306	0.4311	0.4416	0.3607	0.2957	0.0413	0.3076	0.5434	0.1783	****	0.7421	0.6562	0.9567	0.7486	0.8242	0.8690
13	0.1652	0.1578	0.7336	0.4438	1.1201	1.1032	0.0133	0.5544	0.0166	0.1902	0.8663	0.2983	****	0.7459	0.6414	0.9821	0.3955	0.9615
14	0.4531	0.4847	1.2640	0.6175	0.6791	1.9801	0.3558	0.4894	0.2847	0.0895	0.3432	0.4213	0.2932	****	0.4461	0.8270	0.6006	0.8164
15	0.5422	0.5593	0.2539	0.4728	0.4835	0.2113	0.4101	0.0589	0.4602	0.9572	0.2792	0.0443	0.4441	0.8071	****	0.6243	0.7797	0.7714
16	0.1650	0.1626	0.7698	0.3971	0.9476	1.1459	0.0202	0.5146	0.0169	0.1324	0.7288	0.2895	0.0181	0.1900	0.4712	****	0.4325	0.9773
17	1.0462	1.1384	0.5278	0.5383	0.2022	0.3818	1.0102	0.0867	0.9867	0.7480	0.0574	0.1934	0.9275	0.5098	0.2489	0.8381	****	0.5840
18	0.1777	0.1813	0.5489	0.3389	0.6955	0.7587	0.0385	0.2866	0.0411	0.1896	0.4726	0.1404	0.0392	0.2028	0.2596	0.0230	0.5379	****

orestes and Wake County to I = 0.9990 for Watauga and Washington counties (Table 5). Several anomalies suggested that these calculations were sensitive to missing data. For example, Wake and Johnston counties had very similar allelic frequencies for the two loci with data, yet had high genetic differences (D = 0.21). Removing the MPI locus from the data set altered the results (Table 6). Genetic distances calculated using three loci ranged from D = 0.0012 between Watauga and Washington counties to D = 1.98 between Wake and Wilkes counties (Table 6). Similarity values ranged from I = 0.138 for *Desmognathus orestes* and Wake County to I = 0.9990 for Watauga and Washington counties (Table 6).

When genetic distances were used to make the UPGMA/ neighbor joining dendrograms in PopGene, five divergent groups of populations were evident (Figures 7 and 8, respectively). One group of northern and western populations (Group A, green in Figure 7) cluster with the union of the outgroup (*Desmognathus orestes*) and Gaston County (Group B, blue in Figure 7) at D = 0.24 (from GENE). These (A + B = C) all cluster with another group at D = .721. In the alternate part of the dendrogram, Caldwell County (Group E, purple in Figure 7) was the outgroup to five other populations (Group F, red in Figure 7) at D = 0.129. The remaining three populations (Group G, black in Figure 7) cluster with groups F + E at D = 0.40. Branches in Group G joined at D = 0.229, which was greater than apparently longer branches connecting groups E and F.

When the distance calculations were based on three loci, the dendrogram had five different major branches (Figure 8). These five groups differed from those in Figure 6 only for populations which had data for the MPI locus. The same group of northern and western populations (Group A, green in Figure 8) clustered with the outgroup

Figure 7. UPGMA dendrogram for *Desmognathus fuscus* constructed from Nei's (1972) genetic distances for all loci. Neighbor joining tree based on modifications on Neighbor in Phylip. Note that branch lengths do not correspond directly to Nei's distances.

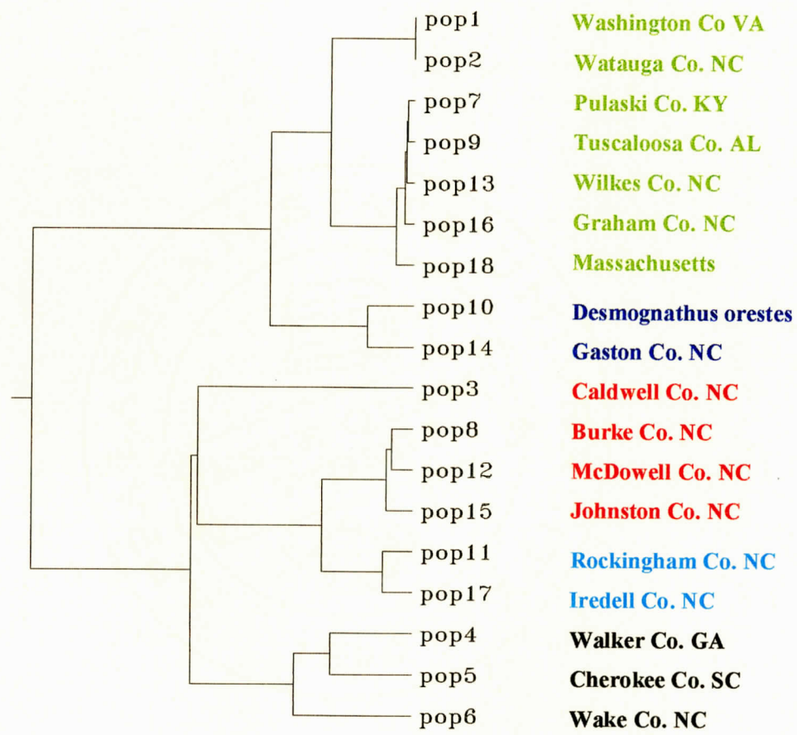


Figure 8. UPGMA dendrogram for *Desmognathus fuscus* constructed from Nei's (1972) genetic distances excluding MPI locus. Neighbor joining tree based on modifications on Neighbor in Phylip.

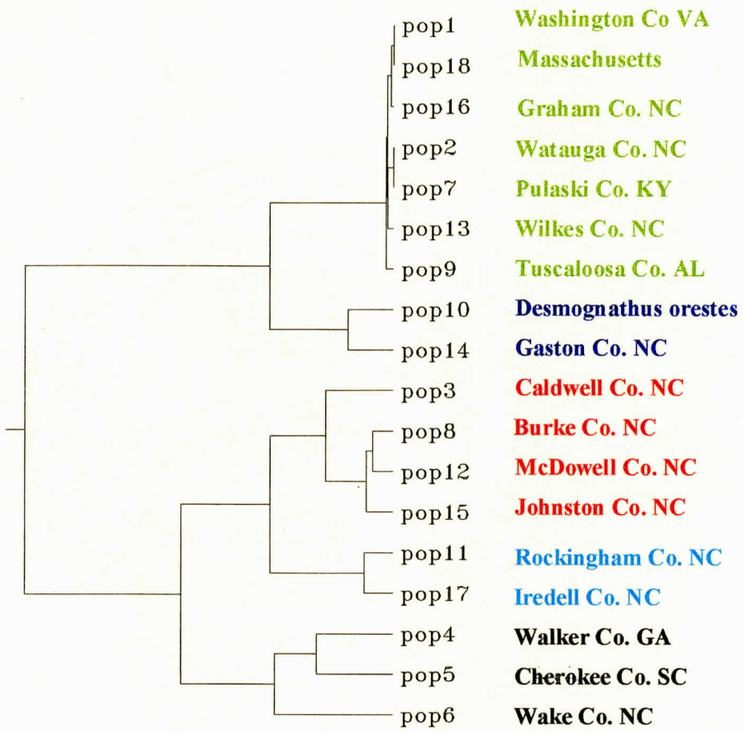
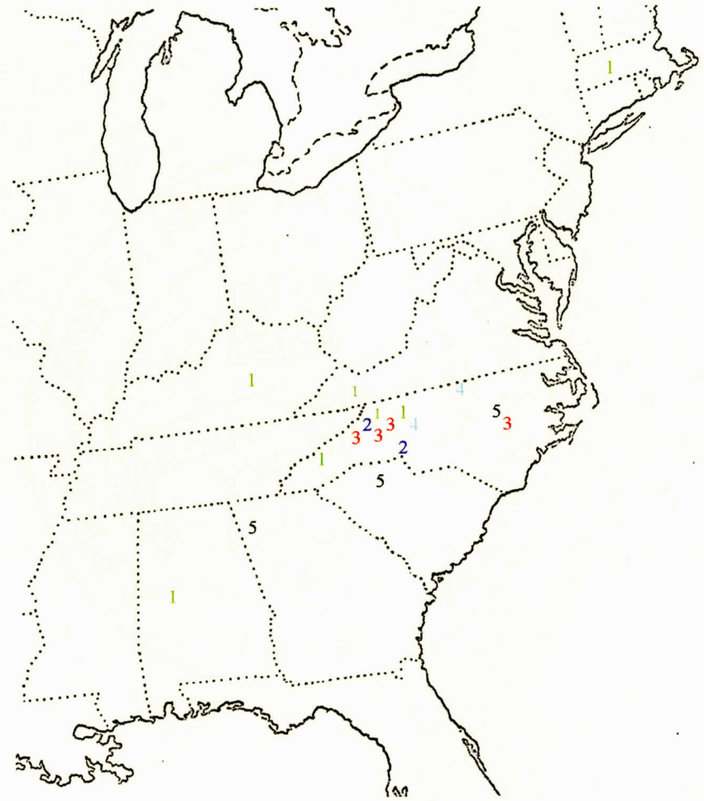


Figure 9. Map of study sites indicating genetic similarity according to Nei's genetic distance. Numbers and colors correspond to groups in dendrogram.



(*Desmognathus orestes*) + Gaston County, NC (Group B, blue in Figure 8) at D = 0.129 (GENE). These (A + B = C) all cluster with another group at D =0 .688. In the alternate part of the dendrogram, a group of four populations (Group E, red in Figure 8) was the outgroup to two other populations (Group F, light blue in Figure 8) at D = 0.231. The remaining three populations (Group G, black in Figure 7) cluster with groups F + E at D = 0.40. Branches in Group G joined at D = 0.229, which was close to the branch lengths connecting groups E and F.

In some cases major branches in Figure 8 corresponded to known biogeographic units (Table 7). Group A (green, Figures 8 and 9) was a widespread unit which extended from Massachusetts through the Appalachian Mountains to Tuscaloosa, Alabama. It also extends somewhat east of the Blue Ridge escarpment. This extensive distance covered a large elevation range, many major river drainages and at least six physiographic provinces (Table 7). This distribution was partially congruent with that of the Seal Salamander (*Desmognathus monticola*) and the Spring Salamander (*Gyrinophilus porphyriticus*) and the Northern Ringneck Snake (*Diadophis punctatus edwardsi*).

Group B (blue, Figures 8 and 9) was a surprise since *Desmognathus orestes* was not as far distant from the rest of the populations as expected. This group included only two populations which differed in elevation, physiographic provinces, and river drainages (Table 7). This pattern was not similar to any known species of amphibian or reptile.

Group C (red, Figures 8 and 9) included a linear swath from two sites at the base of the Blue Ridge escarpment (Caldwell and McDowell counties) through the lower elevations of South Mountain of Burke County to a far eastern site in Johnston County

NC. The northern periphery of several distributions was similar to that of group C, e. g. Eastern Crowned Snake (*Tantilla coronata*) and Green Anole (*Anolis carolinensis*).

Group D (light blue, Figures 8 and 9) was restricted to the central piedmont region of NC and consisted of Iredell and Rockingham counties. Each population occupied its own river drainage and no known reptiles or amphibians were found exclusive to this area.

Group E (black, Figures 8 and 9) occupied a path from northern Georgia to Wake County. Each of the populations (Walker, Wake, and Cherokee) occupied its own river drainage. Two reptiles, Yellow Bellied Slider (*Trachemys scripta*) and Eastern Slender Glass Lizard (*Ophisaurus attenuatus*) share their northern edge of the range with groupE.

Table 8. Geographic characteristics of *Desmognathus* groups. Elevation was categorized as Low (0-239 m), Medium (240-479 m), or High (> 480 m). Physiographic provinces were numbered as recognized in (Griffin, 1965). "Similar Patterns" included species of amphibians and reptiles that share all or part of the range of the population group (Collins and Collins, 1998).

Population Group	Elevation	River drainages for study populations	Similar Patterns	Physiographic provinces
A	H, M, L	7	*D. monticola* *G. porphyriticus* *D. punctatus edwardsi*	6
B	H, M	2	none	2
C	H, M, L	3	*T. coronata* *A. carolininsis*	3
D	M	2	none	1
E	L	3	*T. scripta*, *O. attenuatus*	2

Discussion

The salamanders in this study revealed several interesting patterns of variation at each of the scales studied: gene locus, population, or structured group of distinct genetic entities. Each of the four loci studied reveals a somewhat different pattern of variation. In the four populations with data, the MPI locus has a fixed difference between populations. Fixed differences between populations result from long periods of isolation, intense inbreeding or strong selection (Wright, 1978). It is unclear which of these might be responsible for the unusual allele in Caldwell County.

The MDH2 locus has one wide spread allele and two others restricted to the piedmont of NC. The LGG-PEP locus has a common allele that is mostly found in the northern and western parts of the study area. Wake and Johnston Counties are exceptions. Three rare alleles associated with the LGG-PEP and restricted to the piedmont of NC. The ICD2 locus has a common allele found in the western areas of the study and an alternate common form found in the piedmont of NC. Rare alleles for the ICD2 locus are found in the piedmont of NC.

Within populations alleles are usually in Hardy-Weinberg equilibrium as $F_{IS,}$ which indicates a reduction in heterozygosity associated with inbreeding within a subpopulation. Exceptions to the Hardy-Weinberg equilibrium are usually associated with the LGG-PEP locus and probably reflect the three rare alleles for that gene. (Hartl, 1987).

However, large F_{IT} and F_{ST} values for all loci revealed a large amount of variation among the populations. They were clearly not subsamples of one larger meta population

(Wright, 1978). UPGMA dendrograms (Figures 7 and 8) revealed five groups of populations separated from one another by Nei distances of at least 0.154 A value often used to recognize species level differences in salamanders (Highton, 1989; Tilley 1996; Titus and Larson, 1996).

The key to interpreting levels of divergence between populations is the placement of *Desmognathus orestes* population (population 10). *D. orestes* clusters fairly closely with only the Gaston County, NC population of *D . fuscus* (Nei D = 0.089). This distinction indicates conspecific status in other species but the Gaston County population differs from *D. orestes* in morphology (keeled tail vs. round tail and spotted belly) and is well outside the known range of *D. orestes*. Additional work will be necessary in this area before we can understand whether the Gaston County population represents a completely new species (New species 1) or a range extension of *D. orestes* or the related (and geographically closer) *D. carolinensis*.

A number of closely related populations form Group 1 with Nei's D range from 0.001 to 0.020, values much too small to indicate species level divergence. This group almost certainly includes the type locality for *Desmognathus fuscus* and can be referred to by that name. Group 1 unites with Group 2 at Nei D = 0.24, a level indicating species level divergence (Highton, 1989). Populations in this group are found in or near the Appalachians of North Carolina. Two populations of Group 1 are from lower (> 480 m) elevations (Tuscaloosa and Wilkes Counties).

The cluster of *D. orestes*, New Species 1, and *Desmognathus fuscus* (Group 1) is only distantly related (Nei's D = 0.541) to the other half of the dendrogram. The second half of Figure 8 also includes several divergent groups of populations.

Group 3 (Figures 7 and 8) is composed of populations from the upper piedmont of NC, plus Johnston County NC, which borders the coastal plain. Johnston county is an unusual addition to Group 3 because it is so far from the others (approximately 250 km from the nearest, Caldwell County). Johnston County is closest to the Wake County population (approximately 50 km) and the two sites occupy the same river drainage. Allele frequencies between Wake and Johnston Counties are nearly identical except for a missing set of data at the MDH2 loci, also missing from Walker and Cherokee Counties. Grouping Johnston County with Group 3 and not group 5 may be due to similar missing data and not true genetic difference.

Group 4 from the central piedmont of NC joins Group 3 at a Nei's D of 0.231, a species level divergence between the two groups (Highton, 1989). No name is available for either group so they are probably a New Species 2 and New Species 3. Work is needed to clarify the ranges of the two new species of *Desmognathus* restricted to the coastal plain of NC (New Species 2 and 3).

Populations in the southern areas in the study form group 5 and are separated from New Species 2 and 3 with a Nei's distance of D = 0.40. This is well above species levels of divergence. This group is probably what has been called *Desmognathus conanti* (Rossman, 1958). The type locality for *D. conanti* was not included in this study, but these salamanders are within its range and differ morphologically from the other groups. *D. conanti* is usually brightly colored and spotted with a keeled tail. Based on a great genetic distance (D = .724) between Group 5 (*D. conanti*) and Group 1 (*D. fuscus*), *D. conanti* should not be considered a subspecies of *D. fuscus* but should be recognized as a full species. This conclusion concurs with the work of Titus and Larson (1996) and

Bonett (2002) who, based on mitochondrial DNA and electrophoresis, respectively recommend to elevate *D. f. conanti* to species level. Additional work is required to see if the range of *D. conanti* follows the western edge of the coastal plain of North Carolina into Wake County.

Elevation seems to be the defining variable to understanding *Desmognathus* population distributions. Populations for *D. fuscus* are found at higher elevations (χ = 557 km) than populations for *D. conanti* and New Species 1, 2 and 3 (χ = 278 km). River drainages, physiographic provinces and distributions of similar organisms do not define the ranges of the salamander groups as clearly as elevation.

Work in several areas will better characterize these populations. An established contact zone between New Species 1 and *Desmognathus fuscus* could show elevation tolerances of the two groups. Research could demonstrate why Wilkes County has *Desmognathus fuscus* and nearby Caldwell County has New Species 1 or why the upper piedmont has such a relatively high proportion of rare alleles. Home ranges and population densities of each new species must be known for possible conservation efforts. Does the piedmont of North Carolina, or the piedmont of other states have additional species?

The sacrifice of 756 salamanders and many hours of analysis has revealed the existence of three new species of *Desmognathus* in North Carolina. Descriptive efforts will follow.

LITERATURE CITED

Barbour, R. W. 1950. A new subspecies of the salamander, Desmognathus fuscus. Copeia 1950(4):277-278.

Bishop, S. C. 1947. Handbook of Salamanders. The Salamanders of Canada, and of Lower California. Hafner Publishing Co., New York.

Bonett, R. M. 2002. Analysis of the contact zone between the dusky salamanders *Desmognathus fuscus fuscus* and *Desmognathus fuscus conanti* (Caudata: Plethodontidae). Copeia 2002(2):344-355.

Conant, R. 1959. A Field Guide to Reptiles and Amphibians of the United States and Canada east of the 100th Meridian. Houghton Mifflin Co., Boston.

Conant, R., and J. T. Collins. 1998. A Field Guide to Reptiles and Amphibians of Eastern/Central North America. 3rd Edition, Expanded. Houghton Mifflin Co., New York

Dunn, E. R. 1926. The Salamanders of the Family Plethodontidae. Smith College 50th Anniversary Publication, Northampton, Massachusetts.

Frost, D.R. 2002. Amphibian species of the world: an online reference. V2.21 (15 July 2002). Electronic database available at: http//research.amnh.org/herpetology/amphibia/index/html.

Grobman, A. B. 1950. The distribution of the races of *Desmognathus fuscus* in the southern states. Natural History Miscellanea 70:1-8.

Griffin, J. B. 1965. Late Quaternary prehistory in the northeastern woodlands. Pp. 655-671 In: Wright, H. E., Jr., D.G. Frey. Eds. The Quaternary of the United States. A Review Volume for the VII Congress of the International Association for Quaternary research. Princeton University Press, Princeton New Jersey.

Hartl, D. L. 1988. A Primer of Population Genetics, 2nd edition. Sinauer Associates, Inc. Sunderland Massachusetts.

Highton, R. 1977. Comparison of microgeographic variation in morphological and electrophoretic traits. Evolutionary Biology 10:397-436.

Highton, R., G. C. Maha, L. R. Maxon. 1989. Biochemical evolution in the slimy salamanders of the *Plethodon glutinosus* complex in the eastern United States. Part I. Illinois Biological Monographs 57:1-78.

Highton, R. 1989 Biochemical evolution in the slimy salamanders of the Plethodon glutinosus complex in the eastern United States. Illinois Biological Monographs 57:1-78.

Highton, R., and R. B. Peabody. 2000. Goegraphic protein variation and speciation of the *Plethodon jordani* and *Plethodon glutinosus* complexes in the southern appalachian mountains with the description of four new species. pp. 31-94 In: Bruce, R. C., R. G. Jaeger, and L.D. Houck. Eds. The Biology of the Plethodontid Salamanders. Kluwer Academic/Plenum publishers, New York.

Hubricht, L. 1985. The distributions of the native land mollusks of the eastern United States. Fieldiana Zoology, New Series No. 24, Publication 1359.

Jacobs, J. F. 1987. A preliminary investigation of geographic genetic variation and systematics of the two-lined salamandes, *Eurycea bislineata* (Green). Herpetologica, 43(4), 1907, 423-446.

May, B., C. C. Krueger, and W. Eng. 1981. GENE computer program.

Means, D. B., and A. A. Karlin. 1989. A new species of *Desmognathus* from the coastal plain. Herpetologica 45(1):37-46.

Nei, M. Genetic distance between populations. American Naturalist 106:238-292.

Petranka, J. W. 1998. Salamanders of the United States and Canada. Smithsonian Institution press, Washington.

Rossman, D. A., 1958. A new race of Desmognathus fuscus from the south-central US. Herpetologica 11:158-160.

Tilley, S. G., R. B. Meritt, B. Wu, and R. Highton. 1977. Genetic differentiation in salamanders of the *Desmognathus ochrophaeus* complex (Plethodontidae). Evolution 32:93–115.

Tilley, S. G., and P. Schwerdfeger. 1981. Electrophoretic variation in Appalachian populations of the *Desmognathus fuscus* complex (Amphibia: Plethodontidae). Copeia 1981(1):109-119.

Tilley, S.G. 1981. A new species of *Desmognathus* (Amphibia: Caudata: Plethodontidae) from the southern appalachian mountains. Occasional Paper of Zoology University of Michigan (659)1-23.

Tilley, S. G., and M. J. Mahoney. 1996. Patterns of genetic differentiation in salamanders of the *Desmognathus ochrophaeus* complex (Plethodontidae). Herpetological Monographs 10: 1-42.

Titus, T. A., and A. Larson. 1996. Molecular phylogenetics of desmognathine salamanders (Caudata: Plethodontidae): a reevaluation of evolution in ecology, life history and morphology. Systematic Biology 45:451-472.

Wright, S. A. 1978. Populations in Nature: a Genetic Approach. Smithsonian International Press, Washington.

Wake, D. B., and E. Jockush. 2000. Detecting species borders using diverse data sets, examples from plethodontid salamanders in California. pp. 95-121. In: Bruce, R. C., R. G. Jaeger, and L. D. Houck. Eds. The Biology of the Plethodontid Salamanders. Kluwer Academic/Plenum publishers, New York.

Yeh, F. C., R. Yang, and T. Boyle. 1999. PopGene computer program.

Appendix 1

Locality data for all *Desmognathus* study sites

Specimens collected are listed alphabetically by state followed by COUNTY, (population number), locality and ASU reptile, mammal, and amphibian collection numbers. Counties with specimens not electrophoretically analyzed lack population numbers and counties marked *** do not have ASU collection numbers.

Alabama: TUSCALOOSA County (9) Tuscaloosa, seep behind Lake Nichol, ASU # 21258- 21268; 21989-22030.

Georgia: WALKER County (4) Pigeon Mountain, Rocky lane near check station, ASU # 20037-2078.

Kentucky: PULASKI County (7) Bee Rock campground, Hwy. 192, ASU # 20331-20369.

Massachusetts: HARTFORD County (17) Horse Mountain, ASU # ***

North Carolina: ALLEGANY County, SR 1562 0.3 miles E. SR 1461, 20801-20807; 20837-20848; 21001-21009. ASHE County, SR 1575, 0.65 miles N. SR 1576, ASU #***. BURKE County (8) South mountain, ASU # 21116-21152. CALDWELL County (3) Globe Rd. 3.2 miles SSE Watauga county line ASU # 19945-19953; 20123-20142; 20201; 20325-20330. GASTON County (14) Stanley, Old Hwy. 27 at Sandy Ford Rd., ASU # 20468-20481; 21084-21097. GRAHAM County (16) Stecoah Gap, ASU # 21049-21073. GUILFORD County, Hwy. 68 at Harrell Rd., ASU # 21271-21278. IREDELL County (17) SR 2362 (Triplett Rd.) 0.3 miles E. of SR 2379, ASU # 20601-20618; 20628-20639. JOHNSTON County (15) Smithfield, SR 1562 0.5 miles S. SR 1563, ASU # ***21103-21115. LINCOLN County, HWY. 27 0.36 miles W SR 1002, ASU # 21077-21081; 21083. MCDOWELL county, (12), Hwy. US 221 just S. of Johnston Hollow Rd., ASU # 21018-21020; 21041-21049; 20495-20502. MECKLINBURG County, Hwy. 115 0.28 Miles N. of Eastfield Rd. ASU # 20300-20322; ***. RANDOLPH County, SR 1188 0.3 miles S SR 1119, ASU # 20550-20551; 21021-21040. ROCKINGHAM County (11) Hwy. 29 1.2 mi. N. Hwy. 87, ASU # 20482-20491;***. ROWAN County, SR 1002 0.7 miles N. SR 2134, ASU # 20808-20815; 20547-20550; 20552-20556. SURRY County, Franklin Rd. 3.3 miles W Hwy. 89, ASU # 20210-20222; 20268-20281. WAKE County,(6) SR 1562 0.3 miles E SR 1461, ASU # 20824-20830; 21290-21300; 22031-22031. WATAUGA County, (2), Boone, Hidden Valley Circle Rd. ASU # 20236-20248; 20323-20324; 21189-21200; 21201-21230; 21290-21300. WATAUGA County (10) Boone, ASU Environmental studies area (Outgroup *Desmognathus orestes*. WILKES County (13) Briarwood Rd. at Rivers St. 1.6 miles W.intersection Hwy 16 and 421.

South Carolina: Cherokee county, (5), Hwy, 221-S, 1 mile south of NC border, # 20561- 20572; 21020; 21082; 21084-21098.

Virginia: Washington county, (1), Hayter's Gap, Hwy. 80, 1 mile south of Holston River. ASU #

Appendix 2

Gel Buffer, Electrode Buffer and Protein Stain Protocol

Bath and Gel Buffers for *Desmognathus* Electrophoresis

Lithium hydroxide system

Solution A (pH 8.3)

LiOH	7.20 g
Boric acid	71.34 g
Water to 6 l	

Solution B (pH 8.4)

Citric acid monohydrate	9.60 g
Tris	37.30 g
Water to 6 l	

BATH BUFFER: Solution A, full strength
GEL BUFFER: 40 ml A, diluted to 400ml with B
Run gel at 45 mA for 18 hours

Poulik system

BORATE BATH BUFFER (pH 8.2)

Boric acid	111.30 g
NaOH	14.40 g
Water to 6 l	

Use full strength

POULIK GEL BUFFER (pH 8.7)

Tris	55.26 g
Citric acid monohydrate	6.30 g
Water to 6 l	

Run gel at 45 mA for 18 hours

Tris citrate system (pH 6.7)

TRIS CITRATE 6.3 BATH BUFFER

Tris	162.00 g
Citric acid monohydrate	108.42 g
Water to 6 l	

Adjust pH with 4 M and 1 M NaOH
Use full strength

TRIS CITRATE 6.7 GEL BUFFER STOCK SOLUTION

Tris	30.98 g
Citric acid monohydrate	20.16 g
Water to 4 l	

Adjust pH with 4 M and 1 M NaOH
Use 50 ml diluted to 400 ml with water
Run gel at 20 mA for 18 hours

Tris citrate system (pH 8.0)

Tris	416 g
Citric acid monohydrate	165 g
Water to 5 l	

BATH BUFFER: Use full strength
GEL BUFFER: Use 13.3 ml diluted to 400 ml
Run gel at 60 mA for 18 hours

Karlin's TBE system (pH 9.1)

Tris	50.90 g
EDTA	16.20 g
Boric acid	0.25 g
Water to 5 l	

Use full strength for bath and gel.
Run gel at 45 mA for 18 hours

Tris versine borate system (pH 8.0)

Tris	363.00 g
Boric acid	240.00 g
EDTA	36.00 g
Water to 6 l	

GEL BATH: Use full strength.
GEL BUFFER: 40 ml diluted to 400ml.

Stain Buffers for *Desmognathus* Electrophoresis

0.2 M TRIS HCL (pH 8.0)

Tris	145.24 g
Concentrated HCL	60.00 ml
Water to 6 l	

0.5 M TRIS HCL (pH 7.1)

Tris	60.55 g
Concentrated HCL	37.50 ml
Water to 5 l	

0.5 M TRIS HCL (pH 7.5)

Tris	60.55 g
Concentrated HCL	30.00 ml
Water to 1 l	

0.5 M PHOSPHATE BUFFERS

Stock solution 1:	
K2HPO4 (3H2O)	57.05 g
Water to 500 ml	
Stock solution 2:	
K2HPO4	34.00 g
Water to 500 ml	

Titrate to desired pH.

Stain Protocols for *Desmognathus* Electrophoresis

0.2 TRIS HCL (pH 8.0) BUFFER

System	Buffer	0.1 M MgCl 2	1% NAD	1% NAPD	1% MTT	1%PMS	Other Ingredients
ACON	34ml	1.0 ml		0.4 ml	1.0 ml	0.5 ml	Sub.sol.....3.7 ml
		1.0 M					IDH.....8 mg
AK	15ml	6.0 ml	2.0ml		0.5 ml	0.5 ml	Glucose...100mg
							ADP.....30mg
							Hexokinase.80ug
							G6PDH.....20ug
							Agar.....0.3g
							Buffer.....15ml
AAT	20ml						Sub. sol.....20ml
							Fast garnet.80mg
FUM	40 ml		0.8 ml		1.0 ml	0.5 ml	Fum. Acid..0.04g
							MDH.....120ug
							Na pyruv....10mg
GPI	30 ml	10 ml	1.0 ml		1.0 ml	0.5 ml	Fruc.-6-P...22mg
							G6PDH.....56ug
GDH			3 ml		1 ml	0.5 ml	Glucose.....10 g
							.5M Phosp..40 ml
ICD	33 ml	2.5 ml		0.8 ml	1.0 ml	0.5 ml	Sub. sol....2.0 ml
AGPDH	37mL	0.8 ml	1.6 ml		0.5 ml	0.5 ml	A-glycero.530mg
							Na pyruv.163 mg
LDH	33 ml		1.8 ml		1.0 ml	0.5 ml	Sub. sol.....3.5ml
MDH	34 ml	1.5 ml	1.7 ml		1.0 ml	0.5 ml	Sub. sol....2.0 ml
ME	34 ml	1.5 ml		1.7 ml	1.0 ml	0.5 ml	Sub. sol....2.0 ml
MPI	38 ml		0.8 ml		0.5 ml	0.5 ml	Mannose6P38mg
							Na Pyruv...40 mg
							GPI.....38ug
							G6PDH.....15ug
6PGDH	5 ml	5.9 ml	0.1 ml		1.0 ml	0.1 ml	6- phospho-gluconate...10mg
SOD	35 ml	3.5 ml			1.0 ml	0.5 ml	

0.1 M TRIS HCL (pH 7.0) BUFFER

CK	15 ml	5.0 ml	1.2 ml	1.0 ml	0.5 ml	Dextrose...45 mg
						Creatine-Phosphate365 mg
						ADP.....35 mg
						Hexokinase80 ug
						G6PDH.....40 ug
						Agar.....0.3ml
						Water.....15 ml

0.5 M TRIS HCL (pH 7.1) BUFFER

G3PDH	8 ml	2.0 ml	1.0 ml	0.1 ml	Water.....28 ml
					Na arsenat.61 mg
					Fructose 1,6 Diphosph185 mg
					Aldolase...40 mg

0.5 M TRIS HCL (pH 7.5) BUFFER

PEP	15 ml				Leu-gly-gly20 ml
					Snake Ven...10ml
					Peroxidase...20ml
					O-dianisidie10ml
					Agar.....0.3 ml
					Water.....15ml

0.5 M PHOSPHATE (pH 7.5) BUFFER

HBDH	7 ml	0.2 ml	0.4 ml	1.0 ml	0.5 ml	Water.....30 ml
						Hydroxybutyric-acid.....200 ml
						Na Pyruvat.40mg
						20% NaCl.1.2mg

0.5 M PHOSPHATE (pH 7.0) BUFFER

GLUD	8 ml	2.3 ml	1.0 ml	0.5 ml	Water.....27 ml
					Sub. sol....2.0 ml
					Na Pyruvat.40mg

Stain Buffers for *Desmognathus* Electrophoresis

0.2 M TRIS HCL (pH 8.0)

Tris	145.24 g
Concentrated HCL	60.00 ml
Water to 6 l	

0.5 M TRIS HCL (pH 7.1)

Tris	60.55 g
Concentrated HCL	37.50 ml
Water to 5 l	

0.5 M TRIS HCL (pH 7.5)

Tris	60.55 g
Concentrated HCL	30.00 ml
Water to 1 l	

0.5 M PHOSPHATE BUFFERS

Stock solution 1:	
K2HPO4 (3H2O)	57.05 g
Water to 500 ml	
Stock solution 2:	
K2HPO4	34.00 g
Water to 500 ml	

Titrate to desired pH.

Appendix 3

Allele frequencies for *Desmognathus*

Appendix 3. Allele Frequencies for *Desmognathus.* Bold frequencies (population 10) indicate outgroup *D. orestes*.

Locus	Population																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
ICD II																		
1	4	0	90	46	75	100	0	100	0	**0**	100	76	0	13	1	0	1	25
2	0	0	4	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0
3	95	100	6	54	25	0	1	0	100	**0**	0	24	92	86	0	0	0	75
4	0	0	0	0	0	0	0	0	0	**100**	0	0	8	2	0	1	0	
MDH II																		
1	100	100	56	0	0	0	100	100	0	**71**	1	98	94	1	1	1	65	1
2	0	0	44	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	**29**	0	2	6	0	0	0	35	0
MPI																		
1	0	0	100	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0
2	100	100	0	100	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0
LGG																		
1	0	0	0	0	8	0	0	0	19	**29**	10	0	0	7	0	0	3	0
2	8	2	2	36	58	13	0	43	2	**50**	80	20	5	88	3	25	65	25
3	92	98	98	64	35	87	100	57	79	**21**	10	63	78	7	97	75	25	75
4	0	0	0	0	0	0	0	0	0	**0**	0	17	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	8	0

VITA

William Minor Hicks was born in Durham, North Carolina on May 19 1969. He graduated from Greensboro Day School in 1987 and earned a Bachelor of Science in Finance, Insurance and Real Estate from Appalachian State University in May 1992.

In August 1996, he began work on a Master of Science degree in Biology at Appalachian State University. He has co-authored a professional paper regarding butterfly habitat use and is currently employed as an Apiary inspector employed by the North Carolina Department of Agriculture and Consumer Services. He lives in Roxboro, North Carolina with his wife Kim and his son Eli.